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Co-coating of receptor-targeted drug nanocarriers with anti-phagocytic moieties enhances specific tissue uptake versus non-specific phagocytic clearance

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Abstract

Nanocarriers (NCs) help improve the performance of therapeutics, but their removal by phagocytes in the liver, spleen, tissues, etc. diminishes this potential. Although NC functionalization with polyethylene glycol (PEG) lowers interaction with phagocytes, it also reduces interactions with tissue cells. Coating NCs with CD47, a protein expressed by body cells to avoid phagocytic removal, offers an alternative. Previous studies showed that coating CD47 on non-targeted NCs reduces phagocytosis, but whether this alters binding and endocytosis of actively-targeted NCs remains unknown. To evaluate this, we used polymer NCs targeted to ICAM-1, a receptor overexpressed in many diseases. Co-coating of CD47 on anti-ICAM NCs reduced macrophage phagocytosis by ~50% up to 24 h, while increasing endothelial-cell targeting by ~87% over control anti-ICAM/IgG NCs. Anti-ICAM/CD47 NCs were endocytosed via the CAM-mediated pathway with efficiency similar to (0.99-fold) anti-ICAM/IgG NCs. Comparable outcomes were observed for NCs targeted to PECAM-1 or transferrin receptor, suggesting broad applicability. When injected in mice, anti-ICAM/CD47 NCs reduced liver and spleen uptake by ~30–50% and increased lung targeting by ~2-fold (~10-fold over IgG NCs). Therefore, co-coating NCs with CD47 and targeting moieties reduces macrophage phagocytosis and improves targeted uptake. This strategy may significantly improve the efficacy of targeted drug NCs.

GRAPHIC ABSTRACT

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Author Contributions

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Declaration of Interest

The authors declare no conflict of interest.

Surface functionalization of drug nanocarriers with both CD47 and targeting moieties provides anti-phagocytic properties for reduced nonspecific clearance while enabling specific receptor-mediated uptake by target tissue cells.

Keywords

Targeted drug nanocarriers; Receptor-mediated endocytosis; Phagocytic clearance; CD47; Biodistribution

INTRODUCTION

Nanomaterials whose composition and functionalization are compatible with biological systems represent promising tools to access and interact with cells and tissues.¹ An example is that of biomaterials explored for delivery of pharmaceuticals: a myriad of drug nanocarriers (NCs) exist which offer valuable control over drug solubility, stability, release, etc., improving the therapeutic outcome.^{2–6} However, the site-specific targeting of these systems has proven difficult to control since natural clearance mechanisms often eliminate them prematurely, hindering their ability to access the intended body sites.^{2,7,8} For instance, many drug NCs use passive extravasation across capillary pores or gaps to reach tissues, such as the case for numerous NCs explored for cancer therapies.^{5,7,8} In other cases, they are designed to target endothelial-surface receptors involved in active transport between the bloodstream and the underlining tissue,^{9,10} as for those intended to cross the blood-brain barrier.^{11,12} In both cases, NC removal via renal filtration (if sufficiently small) or the mononuclear phagocyte system in the liver and spleen (most commonly) is in detriment of their accumulation in the intended tissue.^{7,8,13} In addition, their removal by tissue resident macrophages also hinders their ability to reach the intended cells.¹⁴ Coupling of drug NCs to affinity molecules such as antibodies, peptides, aptamers, etc. helps them bind and enter target cells, but this does not preclude nonspecific clearance.^{9,15} In fact, some of these affinity moieties may enhance clearance, e.g., through Fc domain of targeting antibodies which can bind to Fc receptors (FcR) on phagocytic cells, nucleic acid-based interaction with scavenger receptors, etc.¹⁴

In order to improve this aspect, grafting of polyethylene glycol (PEG) onto drug NCs is a common strategy to lower interactions with phagocytes and other elements involved in clearance, including opsonins, the complement, etc.^{8,13} However, while undoubtedly valuable, PEG has been associated with generation of anti-PEG antibodies, which defeats this purpose, and its presence also reduces intended targeting interactions.^{9,16} Alternative options to lower phagocytic uptake of nanomaterials include the use of high aspect ratio shapes to promote circulation in the blood midstream¹⁷ and reduce phagocytic uptake, which

depends on the length and stiffness of the device, the angle of contact with phagocytes, etc.¹⁸ Nevertheless, high aspect ratio objects often exceed the dimensions permissible for active vesicular transport across the endothelium or into tissue cells, and how these parameters affect specific routes is unclear.⁹ Therefore, there is significant room for new options which may offer improvement.

An attractive alternative is that of coupling CD47 on the surface of drug NCs. CD47 is a transmembrane glycoprotein expressed on most cells, which plays a key role on the non-phagocytic versus phagocytic balance, among other functions.¹⁹ As an example, red blood cells (RBCs) continuously pass through the hepatosplenic system without being removed, despite the fact that RBCs come in contact with phagocytic cells in these organs.²⁰ This anti-phagocytic effect results from the interaction between the extracellular domain of CD47 expressed on RBCs and the extracellular domain of signal regulatory protein α (SIRP α) expressed on macrophages.^{19,21} Binding of CD47 to SIRP α recruits tyrosine phosphatase SHP-1 to the phagocyte plasmalemma, which then dephosphorylates substrates involved in the phagocytic process.^{20,22} Particularly, phosphorylation of myosin-IIA is required for reorganization/contraction of cytoskeletal acto-myosin elements involved in phagocytic engulfment; hence, myosin-IIA dephosphorylation impairs this process.²³ Thereafter, RBCs detach from phagocytes, which is speculated to depend upon trans-endocytosis of the CD47-SIRP α complex and/or actin polymerization into protrusions that “push” the RBC away.^{24,25} With time, CD47 becomes oxidized and suffers conformational changes on the RBC surface, losing this anti-phagocytic function and resulting in phagocytic removal of senescent RBCs in the spleen.²⁶ This is not limited to CD47 expressed on RBCs: CD47 is rather ubiquitously expressed and, because of this, it acts as a marker of the self and exerts such anti-phagocytic function generically. Such is the case for interactions of phagocytes with CD47-expressing platelets, lymphocytes, tumor cells, hematopoietic stem cells, etc.²⁰ Owing to this, CD47 function is being explored for diverse treatments, e.g., through disruption of CD47-SIRP α interaction between phagocytes and cancer cells or modulation of tissue graft rejection.^{27,28}

In the context of drug delivery,²⁹ murine macrophage cell cultures incubated in the presence of soluble CD47 were observed to exert lower phagocytosis of colloidal drug carriers added thereafter.³⁰ In addition, either the ectodomain of CD47 or specific peptides derived from its SIRP α -binding region have been coupled to the surface of micro- or nano-devices, which has then been shown to reduce phagocytosis and clearance of said devices both in cell cultures and laboratory animals.^{14,23,31} A similar CD47-grafting strategy was applied to the surface of biomaterials used in implants, where it helped reduce neutrophil and macrophage attachment.²⁸ Therefore, CD47 coating is an attractive alternative to lower phagocytic clearance of drug NCs.

Nevertheless, previous pioneer works had focused on devices which were not actively targeted against cell-surface receptors meant to achieve specific binding and induction of endocytosis by target cells. Co-coating of CD47 and affinity moieties used for targeting, e.g., antibodies, could impact the anti-phagocytic activity of CD47 and/or the induction of specific endocytic transport by the intended cells. For instance, presence of targeting antibodies on CD47-coated NCs could exacerbate FcR-mediated signaling toward phagocytosis by macrophages, dendritic cells, etc., since this process finely depends on the

balance between pro-phagocytic and anti-phagocytic signals.¹⁴ In some other cases, phagocytic cells themselves express common targets selected for drug delivery, such as transferrin receptor, folate receptor, etc.^{32,33} Hence, said specific interaction of NCs with macrophages are likely to drive uptake via non-phagocytic routes, e.g., clathrin or caveolar-like endocytosis associated to these receptors.^{12,34} Additionally, although CD47 exerts its anti-phagocytic signal on macrophages by binding to SIRP α , which is not expressed on most tissue cells, CD47 can also bind to other surface proteins, including thrombospondin, integrins, etc.²⁰ Since these molecules are present on other body cells, CD47 may negatively impact the intended endocytosis induced by targeting moieties on NCs. Therefore, both the effect of targeting moieties on CD47 anti-phagocytic function, and the effect of CD47 on the endocytic function of targeting moieties is unknown and unpredictable.

The goal of this study was to examine whether co-coating of CD47 with targeting moieties on the surface of drug NCs would provide reduced phagocytosis by macrophages involved in clearance while still enabling specific binding and uptake by target cells. Our results in cell culture and *in vivo* support this hypothesis.

METHODS

Antibodies and Reagents

Monoclonal antibodies recognizing human or murine intercellular adhesion molecule 1 (ICAM-1), a cell-surface receptor overexpressed in many diseases,³⁵ were from hybridomas from American Type Culture Collection (Manassas, VA). Recombinant human or murine CD47, tagged with poly-histidine or human Fc, were from LD Biopharma Inc. (San Diego, CA) and Creative Biomart (Shirley, NY), respectively. Antibodies to thrombospondin-1 (TSP-1) and integrin $\alpha_v\beta_3$ were from ThermoFisher Scientific (Waltham, MA) and R&D systems (Minneapolis, MN) respectively. Non-specific murine IgG and secondary antibodies (Texas Red-and Alexa Fluor 350-labeled goat anti-mouse IgG) were from Jackson Immuno Research (West Grove, PA). Bovine serum albumin (BSA), herein referred to as albumin, was from Fisher Scientific (Kerrville, TX). Green fluorescent Fluoresbrite®-labeled polystyrene beads of 100 nm or 1 μ m in diameter were from Polysciences Inc. (Warrington, PA). Poly(D,L-lactide co-glycolic acid) was from Lakeshore Biomaterials (Birmingham, AL). ¹²⁵Iodine (¹²⁵I) and ¹³¹Iodine (¹³¹I) were from Perkin-Elmer (Waltham, MA) and Pierce iodination tubes were from Thermo Scientific (Rockford, IL). Media and supplements for cell culture were from Cellgro (Manassas, VA), Gibco BRL (Grand Island, NY), EMD Millipore Corporation (Billerica, MA) or Sigma Aldrich (St. Louis, MO). All other reagents were from Sigma (St. Louis, MO), unless otherwise noted.

Preparation of coated micro- and nano-particles

PLGA NCs were prepared by nanoprecipitation and solvent evaporation, as in our previous publications.³⁶ An organic phase of acetone containing 19 mg/mL PLGA (50:50 copolymer ratio; 32 kDa average molecular weight) and 1 mg/mL FITC was added under agitation into an aqueous phase, and the emulsion was stirred for 16 h to allow evaporation of the organic solvent. The resulting NC suspension was filtered, dialyzed, and concentrated in a rotary evaporator. Alternatively, polystyrene models were commercial polystyrene microparticles

and nanoparticles (labeled with green Fluoresbrite® were indicated). All microscopy examinations of cellular samples described below were conducted upon cell fixation, which neutralizes all intracellular compartments and enables fluorescence detection of FITC-labeled NCs.

Both PLGA and polystyrene formulations were coated by surface adsorption using established protocols,^{37,38} with either non-specific IgG or anti-ICAM, or 1:1 mass ratios of anti-ICAM and IgG, anti-ICAM and BSA, anti-ICAM and CD47, anti-PECAM and IgG, anti-PECAM and CD47, anti-TfR and IgG, anti-TfR and CD47, or IgG and CD47. At the used concentrations, adsorption has been shown to favor outward display of antibodies.³⁹ Also, random orientation may occur, but this provides a similar batch-to-batch coat and is no different from random chemical conjugation of proteins where the linkage may occur at any of the available protein residues. Briefly, 5400 µg/mL particles and 5 µM total protein were incubated for 1 h at room temperature, then centrifuged at 12,000 rpm for 3 min to remove non-coated proteins, and finally re-suspended in 1% BSA-PBS and sonicated with 20–30 pulses at low power. These formulations have been shown to be stable, including lack of aggregation, adsorption of serum albumin, or protein detachment.^{38,40,41}

Particle characterization (shown in Table 1) encompassed the measurement of the hydrodynamic diameter, polydispersity index (PDI), and ζ-potential by dynamic and electrophoretic light scattering (Zetasizer Nano-ZS90, Malvern Instruments; Westborough, MA), as well as the number of protein molecules (antibodies, albumin, or CD47) on the coat using ¹²⁵I-labeling and/or ¹³¹I-labeling to simultaneously detect 2 proteins on the NC coat as described.³⁸ Scanning and transmission electron microscopy (SEM and TEM) were additionally performed prior to nanoparticle coating and after coating + incubation with serum-containing cell medium.

Cell Cultures

For experiments on phagocytosis, macrophages were isolated from the peritoneal cavity of C57Bl/6 mice under anesthesia and based on IACUC approved protocols and University of Maryland guidelines. Briefly, phosphate buffer saline (PBS) was injected in the peritoneal cavity, followed by abdominal massage and collection of said PBS, which was centrifuged at 300 g for 5 min. The cell pellet was resuspended in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 µg/mL streptomycin, 2 mM glutamine, and plated on glass coverslips in 24 well plates for 2 h. After this time, the cell medium was collected to remove non-adherent cells and adherent macrophages were grown for 16 h at 37 °C, 95% relative humidity and 5% CO₂.

For targeting and endocytosis experiments, human umbilical vein endothelial cells (HUVECs) from Clonetics (San Diego, CA) were grown on 1% gelatin-coated coverslips in 24 well plates, using M199 medium (GibcoBBL, Grand Island, NY) supplemented with 15% FBS, 2 mM glutamine, 15 µg/mL endothelial cell growth supplement, 100 µg/mL heparin, 100 units/mL penicillin and 100 µg/mL streptomycin. Where indicated, cells were treated overnight with 10 ng/mL tumor necrosis factor-α (TNFα; BD Biosciences, Franklin Lakes, NJ) to simulate a pathological condition known to cause ICAM-1 overexpression.^{35,37}

Phagocytosis by macrophages

Macrophages were incubated, at either 4°C or 37°C with 1 μ m (non-labeled) or 200 nm (green fluorescent) polystyrene particles coated either with IgG, IgG/BSA, IgG/CD47, anti-ICAM, anti-ICAM/IgG, anti-ICAM/BSA, or anti-ICAM/CD47. Where indicated, incubations were conducted in a continuous manner or in a pulse-chase mode, i.e., cells were first incubated for 30 min in the presence of NCs to allow binding (pulse), then washed to remove unbound NCs and then incubated for additional times up to a total of 3 h, 5 h, or 24 h (chase). Incubations were in control cell medium or medium containing 0.5 μ M cytochalasin D or 0.1 μ M latrunculin A. Macrophages were then washed and fixed with 2% paraformaldehyde. Cell-surface bound particles were counterstained using Texas Red-labeled secondary antibodies, and 4',6-diamidino-2-phenylindole (DAPI) was used to stain cell nuclei in blue. Samples were analyzed by phase-contrast and fluorescence microscopy using an Olympus IX81 microscope (Olympus, Inc., Center Valley, PA), 40 $\times$ or 60 $\times$ oil immersion objectives (UPlanApo, Olympus, Inc., Center Valley, PA), ORCA-ER camera (Hamamatsu Corporation, Bridgewater, NJ), and SlideBook™ 4.2 software (Intelligent Imaging Innovations, Denver, CO). For micron-size particles, the total number of particles associated to macrophages (as viewed by phase-contrast) and that of Texas Red-counterstained surface-located counterparts were manually counted. Instead, nanoparticles were counted using Image-Pro 6.3 (Media Cybernetics, Bethesda, MD) and an algorithm that provides the total count of green fluorescent objects and that of cell-surface located objects co-localizing with Texas Red fluorescence, from which the internalized objects can be calculated.³⁸

Endothelial expression of CD47 binding molecules

Expression of TSP-1 and integrin $\alpha_v\beta_3$ by TNF α -activated endothelial cells was examined by immunostaining with respective primary antibodies and fluorescently-labeled secondary antibodies, followed by microscopy. Specifically, cell-surface expression was assessed in cells that had been fixed with 2% paraformaldehyde but not permeabilized, so that only cell surface markers were accessible to antibodies. Also, total expression was addressed after fixing and permeabilizing cells with 0.1% triton X-100, so that both cell-surface and internalized markers were accessible. Micrographs were quantified to estimate the fluorescence mean intensity of individual cells, from which the background fluorescence was subtracted.

Endothelial binding and uptake of targeted NCs

Control or TNF α -activated HUVECs were incubated at 4°C or 37°C with either IgG NCs, IgG/CD47 NCs, anti-ICAM NCs, anti-ICAM/IgG NCs, anti-ICAM/BSA NCs, anti-ICAM/CD47 NCs, anti-PECAM/IgG NCs, anti-PECAM/CD47 NCs, anti-TfR/IgG NCs, or anti-TfR/CD47 NCs (polystyrene or PLGA, as indicated, both green fluorescent) as described in the respective figures. For mechanistic experiments, incubations were conducted in control cell medium or medium containing 3 mM of amiloride (inhibits CAM-mediated endocytosis), 50 μ M of monodansylcadaverine (MDC, inhibits clathrin mediated endocytosis) or 1 μ g/mL of filipin (inhibits caveolae mediated endocytosis). For experiments discerning involvement of endothelial surface proteins (ICAM-1, TSP-1, and $\alpha_v\beta_3$),

incubations were carried out in the absence (control) or presence (blocking condition) of antibodies against respective cell-surface markers. Also, where indicated, experiments were conducted in a continuous manner or in a pulse-chase mode, i.e., cells were first incubated for 30 min in the presence of NCs to allow binding (pulse), then washed to remove unbound NCs and finally incubated for additional time up to a total of 1 h, 3 h, or 5 h (chase). Cells were then washed, fixed with 2% paraformaldehyde, and incubated with Texas Red secondary antibody to counterstain surface-bound NCs and DAPI for cell nuclei. The microscopy settings and algorithm described above was used to count total NCs (all objects with green fluorescence) and those on the cell surface (green objects co-localizing with red fluorescence, hence yellow), from which the number of internalized NCs and their relative uptake (percent of all cell-associated NCs) was calculated.³⁸

Intracellular trafficking of NCs

TNF α activated HUVECs were first incubated at 37°C with the fluid-phase marker Texas Red dextran (10 kDa), to label lysosomes as described.⁴² Next, cells were incubated with green-fluorescent anti-ICAM/CD47 NCs for a 30 min pulse to allow binding, washed to remove unbound NCs, and incubated in control cell medium for additional time up to 1 h, 3 h or 8 h. Cells were finally washed and fixed with 2% paraformaldehyde. Fluorescence microscopy was used to automatically quantify the number of green-labeled NCs that co-localized with red-labeled lysosomes.^{42,43}

***In vivo* circulation, biodistribution, and TEM visualization of NCs**

C57BL/6 mice were anesthetized and intravenously (i.v.) injected with either IgG NCs, anti-ICAM/IgG NCs, anti-ICAM/BSA NCs, or anti-ICAM/CD47 NCs (polystyrene or PLGA, where indicated) at 1.8×10^{13} NCs/kg body weight. All NCs contained tracer amounts of ¹²⁵I-IgG on the coat. Blood samples were collected from the retro-orbital sinus at 2, 15, and 30 min after injection, and target (lungs, kidney, heart) or clearance organs (liver and spleen) were collected at sacrifice (30 min). The radioactive content and weight of the samples was determined to calculate circulation, biodistribution, and specificity of the formulations, including the percentage injected dose (%ID), the %ID per gram of organ (%ID/g), or the specificity index (SI), as described.⁴⁰ The latter parameter is computed by calculating the %ID/g of organ divided by the %ID/g of blood (ratio of tissue accumulated vs. circulating amount) for a targeted NC, and then dividing this value for the same parameter extracted for non-targeted control NCs.⁴⁰

Additionally, *in vivo* endocytosis of anti-ICAM/ASM NCs by pulmonary endothelial cells was examined by transmission electron microscopy 3 h after injection. For this, mice were perfused under anesthesia with PBS first and then fixative, followed by collection of the lungs and processing for transmission electron microscopy, as reported.⁴⁴

All animal experiments were performed in accordance with IACUC and University of Maryland regulations and the Guide for the Care and Use of Laboratory Animals of the U.S National Institutes of Health.

Statistics

Data are represented as the mean $\pm$ standard error of the mean (SEM). Each cell culture experiment contained duplicate samples ($\sim 10^5$ cells/sample) and was repeated at least two independent times (total 4 samples). *In vivo* experiments were done using a minimum of 5 mice per condition. Significance was determined using the student's *t*-test assuming a *p* level of 0.05, 0.1 or 0.2 where specified.

RESULTS AND DISCUSSION

Effect of CD47 coat on macrophage uptake of targeted NCs

The goal of this study was to examine whether the anti-phagocytic properties of CD47 could be combined with active targeting of drug NCs. Hence, we first validated our setting to tests phagocytosis using a model well described in the literature, consisting of IgG-coated, micron-sized polystyrene particles²⁵. Using macrophages isolated from the peritoneal cavity of mice, and combining fluorescence and phase-contrast microscopy to distinguish surface-bound (immunostained in red) vs. intracellular sites (visible only by phase-contrast; see Methods), we observed significant phagocytosis of 1 μ m IgG-coated particles at 37 °C but not 4 °C: after a 2 h incubation, 19.3 vs. 0.6 microparticles were internalized per cell, respectively (Supplementary Figure S1A), which represented 77.2% vs. 7.9% of all microparticles associated to macrophages (Supplementary Figure S2). Uptake was inhibited by latrunculin A and cytochalasin D, drugs that impair the actin cytoskeleton reorganization necessary for phagocytosis (52% and 46% reduction, respectively; Supplementary Figure S1A). As expected, co-coating of recombinant CD47 on IgG-microparticles reduced phagocytosis by macrophages to a level similar to that of actin inhibitors: 50% reduction in the absolute number of phagocytosed particles (Supplementary Figure S1B) and 54% reduction in the relative uptake rate (Supplementary Figure S3). This is in agreement with the anti-phagocytic activity of CD47 previously shown using similar polystyrene microparticles,²⁵ whose mechanism involves SIRP α -mediated inhibition of myosin II, lowering the actin-myosin contractility required for uptake.²³ Verifying CD47 specificity, no significant reduction in phagocytosis was seen by co-coating a control protein (albumin) on IgG-microparticles (17% reduction and 5% increase for absolute and relative uptake (Supplementary Figure S1B and Supplementary Figure S3). These set of experiments confirmed our macrophage, phagocytic microparticles, and CD47 models.

Next, we examined whether CD47 could also reduce uptake by macrophages when presented on targeted nanocarriers (NCs) instead of untargeted microparticles. Since previous publications had demonstrated the effects of CD47 using polystyrene nanoparticles as non-targeted NC models,²⁵ we used similar nanoparticles but coated them with targeting antibodies. Because polystyrene is not biodegradable, this allowed us to focus on targeting and uptake without concomitant effects of polymer degradation; however, subsequent experiments described below will focus on biodegradable polymers. As an example of targeting, we coated green-fluorescent polystyrene nanoparticles (see Methods) with antibodies against ICAM-1, a glycoprotein overexpressed on the surface of most cells types in many pathological states.³⁵ We and others have reported the use of ICAM-1 targeting in drug delivery, including protein conjugates, polymer nanoparticles, dendrimers, liposomes,

etc., for various therapeutic and diagnostic applications.^{40,45–50} Our previous studies have characterized anti-ICAM NCs in cell culture and in mice,^{38,40,44,51} providing controls for this study.

Prior to coating, polystyrene NCs had average hydrodynamic diameter of 127 nm, PDI of 0.07, ζ -potential of -44.9 (Table 1), and appeared spherical and monodisperse by SEM (Supplementary Figure S4A). The size, PDI, and ζ -potential increased after coating with anti-ICAM (217 nm, 0.09, -32.5 mV, respectively; Table 1), as expected.³⁸ These models displayed 273 antibodies per NC, maintained their spherical shape and monodispersity despite coating and after incubation with serum-containing media for 3 h (Supplementary Figure S4B), and were internalized well by macrophages within 2 h (231 NCs internalized per cell; Figure 1A–B). Co-coating of control IgG on anti-ICAM NCs reduced, as expected, the average number of anti-ICAM molecules on the NC surface (53% reduction), without significantly affecting the average NC size (198 nm), polydispersity (0.13), or ζ -potential (-27.3 mV; Table 1). These NCs were similarly well internalized by macrophages (232 NCs internalized/cell; Figure 1A–B). This validates non-specific uptake, which may be mediated by the Fc region of antibodies binding to Fc receptors (FcR) on macrophages.⁵² In fact, anti-ICAM NCs and anti-ICAM/IgG NCs displayed similar total numbers of antibody molecules on their surface (273 vs. 241; Table 1). Substitution of IgG with a control protein (albumin) did not significantly change macrophage phagocytosis (202 NCs/cell; Figure 1A–B), consistent with the known role of albumin coatings on this process.⁵³ In contrast, co-coating of CD47 on anti-ICAM NC significantly hindered internalization by macrophages by $\sim 50\%$ vs. control anti-ICAM/BSA NCs, which was effective for up to 24 h (the latest time point tested; Figure 1B–C), without affecting size, PDI, ζ -potential, or number of anti-ICAM molecules (Table 1). Hence, CD47 was able to reduce non-specific uptake of targeted NCs by macrophages.

Although this result agrees well with the effect observed for CD47 or SIRP α -binding peptides coated on non-targeted NCs,¹⁴ the outcome was highly unpredictable for a targeted formulation. In nature, the CD47 expressed on erythrocytes or other cells binds to SIRP α on phagocytic cells, and the resulting anti-phagocytic signal is “balanced” against pro-phagocytic signals, e.g., that resulting from opsonization molecules, oxidized markers, etc., interacting with FcR, scavenger receptors, etc.^{20,28,29} The resulting balance can turn on or off the phagocytic process depending on the “weight” of these two opposed signals.^{20,28,29} While non-targeted NCs coated with CD47 may inhibit phagocytosis, the presence on a NC surface of targeting antibodies, which can interact and induce endocytosis on their own may outweigh this property. In particular, since macrophages express a basal level of ICAM-1³⁵ and binding of NCs to ICAM-1 induces endocytosis,³⁷ whether this pro-uptake signal or the CD47 anti-phagocytic signal would prevail was unknown. These set of experiments demonstrate that the presence of targeting moieties on the drug NC surface does not impair the anti-phagocytic signal of the CD47 co-coat.

Effect of CD47 coat on specific binding and endocytosis of targeted NCs by target cells

Given that CD47 showed the desired effect in reducing the number of antibody-targeted NCs “cleared” by phagocytic cells, we next examined its effects on the specific binding to and

endocytosis by target cells. Since ICAM-1 is abundantly expressed by the endothelium during inflammation, we used endothelial cells activated with TNF α as in previous works^{37,38}. Model, fluorescently-labeled anti-ICAM NCs co-coated with control IgG or albumin showed specific binding to (24 and 57 NCs/cell) and uptake by (16 and 38 NCs/cell) endothelial cells, in contrast to lack of binding and uptake of IgG NCs (0.12 NCs bound and 0.09 NCs internalized per cell; Figure 2A–B). Curiously, anti-ICAM/BSA NCs bound better than anti-ICAM/IgG counterparts, which may be due to the fact that albumin may bind to gp60 on endothelial cells.⁵⁴ Most importantly, co-coating of CD47 on anti-ICAM NCs did not decrease either parameter (50 bound and 37 internalized NCs/cell; Figure 2A–B). Internalization increased with time until full uptake was reached at saturation, regardless of CD47: e.g., 97% and 99% of all cell-associated NCs were internalized within 3 h for anti-ICAM NCs co-coated with control albumin vs. CD47 (Figure 2C). Regression analysis showed similar $B_{\max}$ (127 and 132 NCs/cell) and $t_{1/2}$ for maximal uptake (1.06 and 1.22 h) for both types of anti-ICAM NCs, respectively.

These results were then verified using nanoparticles made of poly(lactic-co-glycolic acid) (PLGA), since this represents a clinically relevant and biodegradable polymer. Our previous studies have shown that anti-ICAM PLGA NCs behave similarly as polystyrene models in terms of specific binding, endocytosis and intracellular effects of therapeutic cargo in cell cultures, as well circulation, biodistribution and cargo delivery in mice.^{44,47,51} As shown in Table 1, coating antibodies and CD47 on PLGA NCs increased their average size, PDI and ζ -potential, resulting in formulations with parameters comparable to polystyrene models: e.g., anti-ICAM/CD47 PLGA vs. polystyrene NCs were 197 vs. 193 nm in diameter, had 0.2 vs. 0.09 polydispersity index, and displayed 138 vs. 118 anti-ICAM antibody molecules and 158 vs. 170 CD47 molecules per NC. Control formulations coated with either ICAM-1 or IgG alone, or control co-coating with IgG and CD47, were all similar for both polystyrene and PLGA counterparts (Table 1).

When incubated with endothelial cells, anti-ICAM PLGA NCs showed increased binding for TNF α activated vs. control cells (57 vs. 9 NCs/cell; Figure 3A–B), which was specific over binding of IgG NCs (9 NCs/activated cell; horizontal line in graph). Expectedly, co-coating of IgG with anti-ICAM at a 1:1 molar ratio reduced to half the number of anti-ICAM molecules displayed on NCs (313 vs. 149 molecules per NC; Table 1), resulting in a proportional decay in cell binding (57 vs. 31 NCs/cell; Figure 3A–B). Yet, the relative efficiency of internalization was not affected (56% and 54% of all cell-associated NCs; Figure 3C). This validates our recent observation that, although the absolute number of internalized NCs depends on binding (see absolute uptake in Figure 3C), the endocytosis rate (% uptake) is independent for each individual NC regardless of the total number of NCs bound on a cell.³⁸

Surprisingly, anti-ICAM/CD47 NCs bound better to endothelial cells than anti-ICAM NCs or anti-ICAM/IgG NCs (e.g., 2.1-fold and 3.9-fold, respectively, for activated cells; Figure 3A–B). This was also observed for polystyrene models (2.1-fold increase in binding of anti-ICAM/CD47 NCs vs. anti-ICAM/IgG NCs; Figure 2B). This could be explained if CD47 would bind to endothelial cells. This may be possible, given that this molecule has other co-receptors apart from macrophage SIRP α . For instance, CD47 expressed on “natural

particles”, such as platelets, binds to thrombospondin-1 (TSP-1) expressed on TNF α -activated endothelial cells.⁵⁵ This interaction cis-activates integrin $\alpha_{IIb}\beta_3$ on platelets, which then binds fibrinogen. Coincidentally, platelet-linked fibrinogen binds to endothelial ICAM-1, which strengthens platelet binding on the endothelium.^{55,56} Hence, it may be possible for anti-ICAM/CD47 NCs to bind not only to endothelial ICAM-1 but also TSP-1, and because both receptors co-function, this may provide enhanced binding. Also, endothelial CD47 interacts with integrin $\alpha_v\beta_3$, but this interaction is in cis, for which this is a less likely target.⁵⁷ In fact, coating of CD47 on IgG NCs enhanced endothelial binding over original IgG NCs by 10-fold in control conditions and by 14-fold in TNF- α activated cells (Supplementary Figure S5). However, even in TNF α -activated cells, the absolute binding of IgG/CD47 NCs was only 12 NCs/cell, which would not account for the 2-fold increase in binding of anti-ICAM/CD47 NCs, which represents a surplus of 33 NCs/cell compared to anti-ICAM/IgG NCs (Supplementary Figure S5 and Figure 2B). These data suggest that CD47 coat enhances binding of NCs, but this requires an initial binding of anti-ICAM to ICAM-1. Yet, this enhanced binding did not increase the uptake rate by cells (50–60% for anti-ICAM/IgG NCs or anti-ICAM/CD47 NCs; Figure 3C), validating that the endocytic process is similarly efficient for each individual NC, regardless of the total number of NCs bound on a cell (see absolute NC uptake in Figure 3C).³⁸

Interestingly, although co-coating of CD47 on targeted (anti-ICAM) NCs reduced phagocytosis by macrophages (Figure 1), it did not affect endocytic uptake by the target cells (endothelial in this case; Figures 2–3), which was true for both polystyrene models and PLGA NCs (Supplementary Figure S6). Based on the fact that CD47 inhibited NC uptake by macrophages despite the presence of a specific “ligand-receptor” signal (anti-ICAM–ICAM-1), and since this occurs by disabling cytoskeletal contribution, it could be expected that specific uptake may also be compromised in the target cells. In particular, endothelial uptake via ICAM-1 requires cytoskeletal reorganization,^{37,58} which could have been compromised by the presence of CD47 and the fact that CD47 can bind to endothelial cells. However, because the endothelial targets of CD47 are distinct from phagocytic SIRP α , this must have disabled CD47 inhibitory effects in these cells vs. macrophages. Therefore, impairing phagocytic clearance without compromising specific endocytosis by target cells is possible by combining CD47 with targeting moieties on the NC surface.

Effect of CD47 coat on the uptake mechanism of targeted NCs in target cells

We next examined if presence of CD47 on the coat of targeted NCs would impact their uptake mechanism or intracellular destination. Anti-ICAM NCs are known to be internalized via a clathrin- and caveolae-independent pathway called cell adhesion molecule (CAM)-mediated endocytosis, which involves the activity of amiloride-sensitive sodium/proton exchanger NHE1.^{37,58} Conforming to an active process of endocytosis, uptake of anti-ICAM/CD47 NCs by endothelial cells was inhibited at 4 °C (80% reduction vs. 37 °C; Supplementary Figure S7A and Figure 4A). In agreement with the CAM pathway, uptake was also inhibited in the presence of amiloride (66% of control; Figure 4A), but not monodansylcadaverine (MDC; 92% of control) or filipin (87% of control), which are inhibitors of clathrin- and caveolin-mediated pathways, respectively.³⁷ None of these

inhibitors altered binding of anti-ICAM/CD47 NCs to cells (84–90% of control), verifying amiloride inhibition only on the uptake process (Supplementary Figure S7B).

To further clarify the mechanism of uptake of anti-ICAM/CD47 NCs, their interaction with other receptors was assessed including TSP-1, a candidate receptor which can bind to CD47 in trans,^{55, 57} and integrin $\alpha_v\beta_3$, which binds to CD47 in cis⁵⁷ and, hence, may not intervene here. After verifying TSP-1 and integrin $\alpha_v\beta_3$ expression in endothelial cells (Supplementary Figure S8), binding and uptake of anti-ICAM/CD47 NCs were studied in the presence of antibodies blocking these markers vs. ICAM-1 (Figure 4B–C). Only antibody to ICAM-1 blocked binding (by 90%), while anti-TSP-1 only partially reduced cell interaction (by 44%), and anti- $\alpha_v\beta_3$ or control IgG had no effect (Figure 4B), suggesting that some binding to TSP-1 is possible. However, as for anti- $\alpha_v\beta_3$ or control IgG, anti-TSP-1 did not reduce the percentage of uptake of anti-ICAM/CD47 NCs by cells (106% of control binding; Figure 4C). While the percentage rate of anti-ICAM/CD47 NCs uptake in the presence of anti-ICAM blocker cannot be reliably obtained given their low binding (Figure 4B), this condition showed no internalized NCs (8 NCs/cell vs. 92 NC/cell in the absence of blocker; not shown). Therefore, although CD47 enhances binding of anti-ICAM NCs to endothelial cells, it does not influence the subsequent endocytic mechanism, which was mediated via the CAM pathway. This result was unexpected, given that presence of CD47 on the NC surface or its binding to endothelial cells could have induced independent uptake routes or could have inhibited the CAM-mediated route. Perhaps this did not occur because endothelial ICAM-1 and CD47 co-receptors contribute to common functions in activated endothelial cells, such as in the context of platelet-fibrinogen attachment on the endothelium described above or the CD47-ICAM-1 interaction seen on activated endothelial cells during leukocyte extravasation.^{55,56,59} In any case, CD47 interaction seemed to contribute to binding alone (not endocytosis), and this required anti-ICAM on the coat (supplementary Figure S5).

Effect of CD47 coat on the intracellular trafficking of targeted NCs in target cells

The previous result would suggest that CD47 may not impact the intracellular destination of targeted NCs, since this largely depends on the uptake mechanism for NCs with same physicochemical properties. To examine this aspect, we investigated the colocalization of intracellular anti-ICAM/CD47 NCs to lysosomal compartments stained with fluorescent dextran (Figure 5A). Lysosomes are a predominant destination for NCs internalized via endocytosis, including anti-ICAM NCs; yet, contrary to clathrin or phagocytic uptake which deliver to lysosomes within minutes, the CAM pathway results in a slow (hours-long) trafficking to this compartment.⁴² Our prior studies have shown that dextran pinocytosed by endothelial cells reaches Lamp-1-positive lysosomes within 5–15 min and, since dextran is not degraded by mammalian cells, it serves as a long-lasting label for this compartment.⁴² When intracellular transport of anti-ICAM/CD47 NCs was examined in this model, we found 29% of all cell-associated NCs colocalized with dextran-labeled compartments by 1 h, which increased up to 75% by 8 h (Figure 5A–B), in agreement with the CAM pathway. Concomitant to lysosomal transport, the total number of PLGA NCs associated with cells decreased, i.e., 50% and 70% reductions were observed 3 h and 8 h after uptake as compared to 1 h (Figure 5C). These experiments were conducted in a pulse-chase mode where NCs

were removed from the cell medium after the first hour of incubation, so that transport of NCs can then be tracked independently of binding (see Methods). Therefore, a decrease with time in the total number of NCs along with an increased colocalization with lysosomes could reflect NC degradation within lysosomal compartments, although this remains to be investigated.

Broad applicability of combining CD47 with receptor-targeted NCs

Having demonstrated our main hypothesis, we examined the potential of this strategy from a broader perspective. First, using the same example of anti-ICAM NCs, we compared two recombinant CD47 forms, once where CD47 was tagged by a tail of poly-histidine residues and one where it was fused to human Fc (Figure 6A and Supplementary Figure S9). In both cases, anti-ICAM/CD47 NCs showed similar binding (50 vs. 66 NCs/cell) and internalization by activated endothelial cells (73% vs. 76% of all NCs associated to cells), suggesting that this species could be modulated if needed without affecting the properties provided by the targeting moiety.

Next, we examined if CD47 would alter targeting and uptake of NCs addressed to alternative receptors. For this purpose, we focused on platelet-endothelial cell adhesion molecule-1 (PECAM-1) and transferrin receptor (TfR). As ICAM-1, PECAM-1 is a CAM expressed by endothelial cells and capable of CAM-mediated endocytosis.^{37,60} Yet, contrary to inducible apical ICAM-1, PECAM-1 is rather abundant at the cell-cell border and is not upregulated during pathology.⁶¹ Instead, TfR is expressed by endothelial and many other cell types, is abundant in apical and basolateral surfaces, and is endocytosed via the clathrin-mediated pathway.⁶² As shown in Figure 6B, presence of CD47 on NCs targeted to PECAM-1 or TfR did not decrease either binding or internalization of these formulations by endothelial cells, compared to anti-PECAM NCs or anti-TfR NCs where the CD47 moiety had been substituted by non-specific IgG (Figure 6B and Supplementary Figure S10). For instance, 85 anti-PECAM/IgG NCs bound to cells within 30 min, of which 46 NCs were internalized, and anti-PECAM/CD47 NCs showed 117% and 137% the binding and uptake of the former formulation. In the case of anti-TfR/CD47 NCs, binding and uptake was 119% and 134% compared to control anti-TfR/IgG NCs. A tendency for an increased interaction with cells was observed for NCs bearing CD47, as in the case of ICAM-1 targeting, which may be due to CD47 interaction with endothelial markers, as described above.⁵⁵ This increase had been more patent for ICAM-1 targeting (Figure 3), perhaps because of the described co-function of ICAM-1 and endothelial CD47 receptors.⁵⁹ In any case, these experiments show that the CD47 strategy may be applied to other targets and routes of endocytic uptake by target cells. This is of high relevance because it suggests that the concept of combining anti-phagocytic signals (CD47 or the like) with specific targeting and endocytic ones (illustrated here by addressing ICAM-1, PECAM-1, or TfR) may be broadly applied.

***In vivo* effects of CD47 coat in the circulation and specific biodistribution of targeted NCs**

The results above suggest that targeted NCs co-coated with CD47 may display enhanced specific interaction with the intended tissues by lowering non-specific removal by phagocytic-clearance sites in the body, such as the spleen and the liver. Hence, we examined this *in vivo*. We focused on anti-ICAM NCs, for which we have historical data. Prior studies

by our group and others have shown that anti-ICAM NCs injected i.v. rapidly accumulate in the lungs, since this organ abundantly expresses ICAM-1 and it contains a significant fraction of body vascular endothelium.^{40,44,45} This was verified here for the formulations used in this study (Figure 7). For instance, using polystyrene models we observed that, although their circulating levels were similarly low 30 min after injection (~5% of the injected dose (ID); Figure 7A), anti-ICAM NCs disappeared from the bloodstream more rapidly than control IgG NCs (9 %ID and 40 %ID, respectively, 2 min after injection). However, this was largely due to ICAM-1 targeting, since the same anti-ICAM NCs circulated similarly as IgG NCs when injected in ICAM-1 knockout mice (49 %ID at 2 min after injection). In fact, a strong lung targeting of anti-ICAM NCs was observed (26 %ID by 30 min; Figure 7B), in contrast to IgG NCs or anti-ICAM NCs injected in ICAM-1 knockout mice, which did not accumulate in this organ (2.2 %ID and 2.0 %ID, respectively). Instead, liver and spleen distribution of anti-ICAM NCs in control mice were lower than for IgG NCs (32% ID vs. 58 %ID for the liver, and 4% ID vs. 5 %ID for the spleen), and for anti-ICAM NCs in ICAM-1 knockout mice (63 %ID in liver and 7.6 %ID in spleen). Negligible amounts were found in the heart in all cases (0.23–0.35 %ID), with kidneys receiving low amounts but somewhat specific for ICAM-1: 1.3 %ID for anti-ICAM NCs vs. 0.6 %ID for IgG NCs and 0.8 %ID for anti-ICAM NCs in ICAM-1 knockout mice. Calculation of the %ID per gram of organ, to normalize biodistribution to organs with very different weights, verified specific lung targeting for anti-ICAM NCs: 141 %ID/g vs. 11 %ID/g for IgG NCs and 10.5 %ID/g for anti-ICAM NCs in ICAM-1 knockout mice (Figure 7C). Instead, IgG NCs rather accumulated in the liver and spleen (47 %ID/g and 63 %ID/g vs. 27 %ID/g and 43 %ID/g for anti-ICAM NCs) similar to anti-ICAM NCs injected in ICAM-1 knockout mice (38 %ID/g in liver and 60 %ID/g in spleen). Therefore, these data suggest that both targeting and clearance contribute to the fast removal of anti-ICAM NCs from the circulation, with the lungs representing a prime site for specific targeting. In accord to previous reports^{44,47,58} and cellular data showing endothelial uptake of anti-ICAM NCs, TEM analysis showed seemingly intact capillary vessels and alveolar spaces in the lungs of mice injected with anti-ICAM NCs, with the majority of visible NCs residing within the vesicular compartment in endothelial cells (Supplementary Figure S11).

After this validation, anti-ICAM NCs were co-coated with CD47 vs. IgG or BSA controls (Figure 8). CD47 did not enhance the circulation of anti-ICAM NCs: only 8–9 %ID of anti-ICAM/CD47 NCs or control counterparts were found in blood by 30 min (Figure 8A). This is an interesting result that differs from previous publications showing circulation enhancement by CD47.²⁵ A major difference here is that anti-ICAM NCs very rapidly and profusely target the lungs (as shown above); hence anti-phagocytic activity in liver and the spleen may not result in a prolonged circulation but rather an improved lung uptake, since more NCs would be available for targeting. In fact, this appears to be the case: in terms of total organ accumulation (%ID; (Figure 8B), CD47 presence on anti-ICAM NCs reduced liver uptake by 28% and 31% compared to anti-ICAM/IgG NCs and anti-ICAM/BSA NCs, respectively, and by 32% and 19% in the spleen, respectively. When quantified as %ID/g to normalize per organ weight (Figure 8C), CD47 reduced accumulation of anti-ICAM NCs in the liver by 48% and 27% compared to anti-ICAM/IgG NCs and anti-ICAM/BSA NCs, and by 39% and 38% in the case of the spleen. The heart and kidneys still received negligible

amounts of anti-ICAM/CD47 NCs (<1.5% ID), as for control anti-ICAM/IgG NCs and anti-ICAM/BSA NCs. Hence, CD47 indeed reduced nonspecific clearance by the liver and spleen. In accord to our hypothesis that reduced clearance would render greater targeting rather than prolonged circulation and due to direct accessibility of endothelial ICAM-1 in the lungs, CD47 enhanced lung targeting of anti-ICAM NCs: 1.9-fold enhancement compared to both anti-ICAM/IgG NCs and anti-ICAM/BSA in terms of %ID (Figure 8B), and 1.8-fold and 1.7-fold enhancement, respectively, for %ID/g (Figure 8C). Since TEM analysis had shown that standard anti-ICAM NCs without CD47 resided in endothelial cells in the lungs without visible signs of macrophage activity in this organ (Supplementary Figure S11), CD47 anti-phagocytic activity may have mostly impacted the liver and spleen (verified by lower uptake), leaving more NCs available to target the lungs. Therefore, CD47 provided considerable improvement to the targeting ability of anti-ICAM NCs even though their circulation was not changed. It is possible that for targets less immediately accessible from the circulation, an increased circulation may be observed, but this remains to be explored.

CONCLUSION

Our results in cell cultures and *in vivo* show for the first time that the anti-phagocytic signal provided by CD47 may be combined with receptor-specific targeting used to induce endocytic signals and endocytic uptake by target cells. Presence of CD47 on the coat of actively targeted NCs reduced phagocytosis by macrophages in culture and hepatosplenic clearance in mice, while it did not lower specific binding or endocytic uptake by the intended target cells in culture and organs *in vivo*. Similar results were observed with regard to the use of various CD47 recombinant forms, several receptors of the same endocytic route, or receptors associated to different endocytic pathways, which suggest a broad application of this strategy. This opens a new avenue to reduce phagocytic clearance of NCs while enhancing their specific binding and uptake by target tissues and cells, which may ultimately improve the specificity of targeted nano-biomaterials used for drug delivery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Amil	amiloride
BSA	bovine serum albumin
CAM	cellular adhesion molecule
Fil	filipin

HUVECS	human umbilical vein endothelial cells
ICAM-1	intercellular adhesion molecule 1
ID	injected dose
ID/g	injected dose per gram of tissue
IgG	immunoglobulin G
MDC	monodansylcadaverine
NCs	nanocarriers
PECAM-1	platelet-endothelial cell adhesion molecule 1
TNFα	tumor necrosis factor alpha
TfR	transferrin receptor

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Figure 1. Effect of CD47 on macrophage uptake of ICAM-1-targeted NCs

A) Visualization and B) quantification of the uptake of 200 nm diameter model NCs consisting of green fluorescent polystyrene nanoparticles coated with anti-ICAM alone or 1:1 mass ratio mixtures of anti-ICAM and IgG, anti-ICAM and bovine serum albumin (BSA), or anti-ICAM and recombinant CD47. Incubations were carried out for 2 h at 37°C with mouse peritoneal macrophages, after which surface-bound NCs were counterstained with Texas Red secondary antibody. This enables quantification of surface-bound NCs (green + red = yellow; arrowheads) vs. internalized NCs (green alone; arrows) by fluorescence microscopy, from which the number of NCs internalized per cell was calculated. C) Similar incubations were carried out allowing a 30 min NC binding pulse

followed by uptake chases in NC-free medium up to 24 h, and staining and quantification as in B). A) Dashed lines indicate cell borders, seen by phase contrast. Blue = DAPI-labeled cell nuclei. Scale bar = 10 μ m. B, C) Data are Mean $\pm$ SEM. *Compares to anti-ICAM NCs, #Compares to anti-ICAM/BSA NCs ($p < 0.05$ by Student's t test).

Figure 2. Effect of CD47 on binding and endocytosis of ICAM-1-targeted NCs by endothelial cells

A) Visualization and B, C) quantification of the uptake by TNF α -activated HUVECs of 200 nm diameter green fluorescent, model polystyrene NCs coated with control IgG or 1:1 mass ratio mixtures of anti-ICAM and IgG, anti-ICAM and bovine serum albumin (BSA), or anti-ICAM and recombinant CD47. Incubations were carried out at 37°C for a 30 min binding pulse, after which non-bound NCs were removed and incubations continued in NC-free medium up to: A, B) 1 h, or C) 5 h. Surface-bound NCs were counterstained with Texas Red secondary antibody to enable quantification of surface-bound NCs (green + red = yellow; arrowheads) vs. internalized NCs (green alone; arrows) by fluorescence microscopy. Data were calculated as Mean $\pm$ SEM of: B) the absolute number of NCs associated and internalized per cell, or C) the relative internalization compared to all cell-associated NCs.

A) Dashed lines indicate cell borders, seen by phase contrast. Blue = DAPI-labeled cell nuclei. Scale bar = 10 μ m. B) *Compares to anti-ICAM/IgG NCs ($p < 0.05$ by Student's t test).

Figure 3. Effect of CD47 on binding and endocytosis of ICAM-1-targeted PLGA NCs by endothelial cells

A) Visualization and B, C) quantification of the uptake by control or TNF α -activated HUVECs of 200 nm diameter green fluorescent, poly(lactic-co-glycolic acid) (PLGA) NCs coated with control IgG vs. anti-ICAM, or 1:1 mass ratio mixtures of anti-ICAM and IgG, or anti-ICAM and recombinant CD47. Incubations were carried out at 37°C for a 30 min binding pulse, after which non-bound NCs were removed and incubations continued in NC-free medium up to 1 h. Surface-bound NCs were counterstained with Texas Red secondary antibody to enable quantification of surface-bound NCs (green + red = yellow; arrowheads) vs. internalized NCs (green alone; arrows) by fluorescence microscopy. Data were calculated as Mean $\pm$ SEM of: B) the absolute number of NCs associated per cell, and C) the relative internalization in terms of the absolute number of internalized NCs compared to all cell-associated NCs. A) Dashed lines indicate cell borders, seen by phase contrast. Blue = DAPI-labeled cell nuclei. Scale bar = 10 μ m. B,C) [#]Compares TNF α to control; ^{*}compares to anti-ICAM/IgG NCs, [!]Compares to anti-ICAM NCs ($p < 0.05$ by Student's t test).

Figure 4. Effect of CD47 on the mechanism of endocytosis of ICAM-1-targeted NCs by endothelial cells

Quantification of the uptake by TNF α -activated HUVECs of 200 nm diameter green fluorescent, model polystyrene NCs coated with anti-ICAM and recombinant CD47 (1:1 mass ratio). A) Incubations were carried out at 37°C for 1 h, in control medium or medium containing inhibitors of endocytosis via CAM (amiloride; Amil), clathrin (monodansylcadaverine; MDC), or caveoli (filipin; Fil). Surface-bound NCs were counterstained with Texas Red secondary antibody to enable quantification of surface-bound NCs by fluorescence microscopy. Data were calculated as Mean $\pm$ SEM of the relative internalization compared to the control (no inhibitor) condition. Solid line indicates the % uptake of control IgG NCs. B, C) Similar incubations were carried out at for 30 min NC binding pulse followed by a 30 min chase in NC-free conditions, in control medium or medium containing antibodies to block ICAM-1, thrombospondin1 (TSP1), integrin $\alpha_v\beta_3$, or non-specific IgG. Surface-bound vs. internalize NCs were counterstained and analyzed as above. Data were calculated as Mean $\pm$ SEM of B) the total number of NCs per cell or C) the relative internalization, all compared to the control (no blocker) condition. Solid line

indicates the control condition. *Compares inhibitors or blockers to control ($p < 0.05$ by Student's t test).

Figure 5. Effect of CD47 on the intracellular trafficking of ICAM-1-targeted NCs by endothelial cells

A) Visualization and B, C) quantification of the fate of 200 nm diameter green fluorescent, PLGA NCs coated with anti-ICAM and recombinant CD47 (1:1 mass ratio) in TNF α -activated HUVECs. First, lysosomes were labeled with Texas Red dextran, then cells were incubated with NCs at 37°C for a 30 min binding pulse, following by washing non-bound NCs and chase incubations up to 8 h. This protocol renders lysosome-localized NCs labeled in green and red (yellow; arrowheads) vs. non-lysosomal NCs which appear green alone (arrows) by fluorescence microscopy. Data were calculated as Mean $\pm$ SEM of: B) the percentage of NCs colocalized with Texas Red dextran-labeled lysosomes, and C) the total number of PLGA NCs per cell. A) Dashed lines indicate cell borders, seen by phase contrast. Scale bar = 10 μ m. B,C) *Compared to 1 h ($p < 0.05$ by Student's t test).

Figure 6. Binding and endocytosis of targeted NCs bearing various CD47 forms or targeting antibodies

Quantification of the binding and endocytosis of 200 nm diameter green fluorescent, model polystyrene NCs by TNF α -activated HUVECs. Incubations were carried out at 37°C for a 30 min binding pulse, after which non-bound NCs were removed and incubations continued up 1 h. Surface-bound NCs were counterstained with Texas Red secondary antibody to enable quantification of surface-bound NCs (green + red = yellow) vs. internalized NCs (green alone) by fluorescence microscopy. Data were calculated as Mean $\pm$ SEM of the number of all NCs associated per cell or those internalized per cell. A) NCs were coated with a 1:1 mass ratio of anti-ICAM and recombinant CD47-poly-histidine or recombinant CD47-human Fc. *Compares CD47-human Fc to CD47-poly-his ($p < 0.05$ by Student's t test). B) NCs were coated with a 1:1 mass ratio of anti-PECAM or anti-TfR with either IgG or recombinant CD47. *Compares antibody/CD47 NCs to antibody/IgG NCs ($p < 0.05$ by Student's t test).

Figure 7. Specific targeting of anti-ICAM1 coated NCs in mice

A) Circulation and B,C) biodistribution of 200 nm diameter, model polystyrene NCs coated with IgG or anti-ICAM, injected i.v. in C57Bl/6 mice (ICAM^{+/+}) or ICAM1 knockout mice (ICAM^{-/-}). All NCs contained tracer amounts of ¹²⁵I-IgG. Blood samples were isolated at 2 min, 15 min, and 30 min after injection, while kidney, heart, liver, lungs, and spleen were collected at sacrifice (30 min). Weight and ¹²⁵I content were determined to calculate the: A, B) percentage of the injected dose (%ID) in circulation or accumulated in an organ, or C) the %ID per gram of an organ (%ID/g). Data are Mean ± SEM. Compares to IgG NCs (**p* < 0.05) or to anti-ICAM NCs in ICAM^{-/-} (#*p* < 0.05).

Figure 8. Effect of CD47 coat on the specific targeting of NCs in mice

A) Circulation and B,C) biodistribution of 200 nm diameter, model polystyrene NCs coated with anti-ICAM mixed at a 1:1 mass ratio with IgG, bovine serum albumin (BSA), or recombinant CD47, 30 min after i.v. injection in C57Bl/6 mice. All NCs contained tracer amounts of ^{125}I -IgG. Blood samples were isolated at 2 min, 15 min, and 30 min after injection, while kidney, heart, liver, lungs, and spleen were collected at sacrifice (30 min). Weight and ^{125}I content were determined to calculate the: A, B) percentage of the injected dose (%ID) remaining in circulation or accumulated in an organ, or C) the %ID per gram of an organ (%ID/g). Data are Mean $\pm$ SEM. Compares to anti-ICAM/IgG NCs (* $p < 0.1$, $^{\delta}p < 0.2$ by Student's t test) or to anti-ICAM/BSA NCs ($^{\#}p < 0.1$, $^!p < 0.2$ by Student's t test).

Table 1

Nanocarrier characterization

Formulation	Size (nm)		PDI		Coating (molecules/NC); Mean $\pm$ SEM		
	Mean	SEM	Mean	SEM	Anti-ICAM	IgG	CD47
<u>Polystyrene</u>							
<i>Non-coated</i>							
<i>IgG</i>	214.2	5.5	0.125	0.021	—	—	—
<i>Anti-ICAM</i>	216.9	2.0	0.093	0.024	—	240 $\pm$ 25	—
<i>Anti-ICAM/IgG</i>	197.8	3.4	0.129	0.021	273 $\pm$ 37	—	—
<i>Anti-ICAM/CD47</i>	193.2	4.8	0.087	0.032	133 $\pm$ 2	108 $\pm$ 2	—
<i>IgG/CD47</i>	193.4	2.8	0.185	0.022	118 $\pm$ 2	—	170 $\pm$ 2
<u>PLGA</u>							
<i>Non-coated</i>							
<i>IgG</i>	196.0	6.6	0.224	0.012	—	—	—
<i>Anti-ICAM</i>	190.2	2.9	0.290	0.013	—	215 $\pm$ 9	—
<i>Anti-ICAM/IgG</i>	192.2	9.9	0.270	0.018	313 $\pm$ 18	—	—
<i>Anti-ICAM/CD47</i>	196.6	7.3	0.206	0.041	149 $\pm$ 12	33 $\pm$ 2	—
<i>IgG/CD47</i>	177.3	6.2	0.210	0.027	138 $\pm$ 10	—	158 $\pm$ 10
					—	54 $\pm$ 7	135 $\pm$ 8

PDI: Polydispersity Index; NC = nanocarrier; SEM = Standard error of the mean